

Survival of Plant Tissue at Super-Low Temperatures V. An Electron Microscope Study of Ice in Cortical Cells Cooled Rapidly

A. Sakai and K. Otsuka

The Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan

Received July 11, 1967.

Summary. Experiments were carried out with cortical cells in twig bark of mulberry trees in winter in order to clarify the mechanism of survival at super-low temperatures with rapid cooling and rewarming. Attention was given to the relation between the existence of intracellular ice crystals and survival.

Cortical cells were cooled rapidly by direct immersion into liquid nitrogen or isopentane cooled at various temperatures. After immersion, they were freeze-substituted with absolute ethanol at -78° . They were then embedded, sectioned and examined under the electron microscope for the presence and distribution of cavities left after ice removal.

Cells were found to remain alive and contain no ice cavities when immersed rapidly into isopentane baths kept below -60° . Those cells at intermediate temperatures from -20° to -45° , were almost all destroyed. It was also observed that many ice cavities were contained in the cells immersed rapidly into isopentane baths at -30° . The data seem to indicate that no ice crystals were formed when cooled rapidly by direct immersion into isopentane baths below -60° or into liquid nitrogen.

The tissue sections immersed in liquid nitrogen were rapidly transferred to isopentane baths at temperatures ranging from -70° to -10° before rapid rewarming. There was little damage when samples were held at temperatures below -50° for 10 minutes or below -60° for 16 hours. No cavities were found in these cells. Above -45° , and especially at -30° , however, all cells were completely destroyed even when exposed only for 1 minute. Many ice cavities were observed throughout these cells. The results obtained may be explained in terms of the growth rate of intracellular ice crystals.

The rapid cooling and rewarming method for maintaining viability at super-low temperatures was first presented by Luyet (2). Many attempts to check this hypothesis have been made by using various materials such as plant cells, microorganisms, blood cells, muscle fibers, etc. Survival was obtained, however, with only a few materials which were especially desiccated (3) or which had been treated with ethylene glycol (4).

Luyet's group (5,6,7) have suggested that damage observed at the higher cooling rates might have been caused by the growth, upon rewarming, of intracellular crystallization nuclei originally formed in the course of rapid cooling to deep temperatures. To test this hypothesis and to clarify the factors contributing to survival, they (5,6,7) studied phase transitions and recrystallization temperatures occurring in the course of rewarming, using water and various aqueous solutions. This rewarming followed immersion in an isopentane bath at -70° . Survival curves, supporting the facts obtained with aqueous solutions were not, however, measured with plant tissues (1,10), although Luyet's group (8,9) studied the formation of intracellular ice at high cooling

velocities which were obtained by direct immersion into an isopentane bath at -150° or by immersion into liquid nitrogen. In the less hardy materials used by them, using the electron microscope, ice cavities were invariably observed within the cells.

It was reported (11,12,15,16) that, in extremely hardy cortical cells of woody plants, viability was maintained when a very rapid cooling was combined with rapid rewarming (4), especially when this was also combined with the prefreezing method (11,12,13,14). Survival curves relating the facts concerning recrystallization demonstrated with aqueous solutions, to survival, were first obtained by Sakai (15,16). The results may be explained in terms of the size of the crystals formed within the cells. At high cooling rates, the ice crystals that form in the cells may be small enough to be innocuous; when rewarming is carried out rapidly, they melt before they have time to grow. All cells remain alive. At intermediate cooling rates, survival rates remain very low even when rewarmed rapidly. The ice crystals that form in the cells during cooling may be large enough to be immediately damaging. At low cooling rates, the crystals should be still larger, but they may form

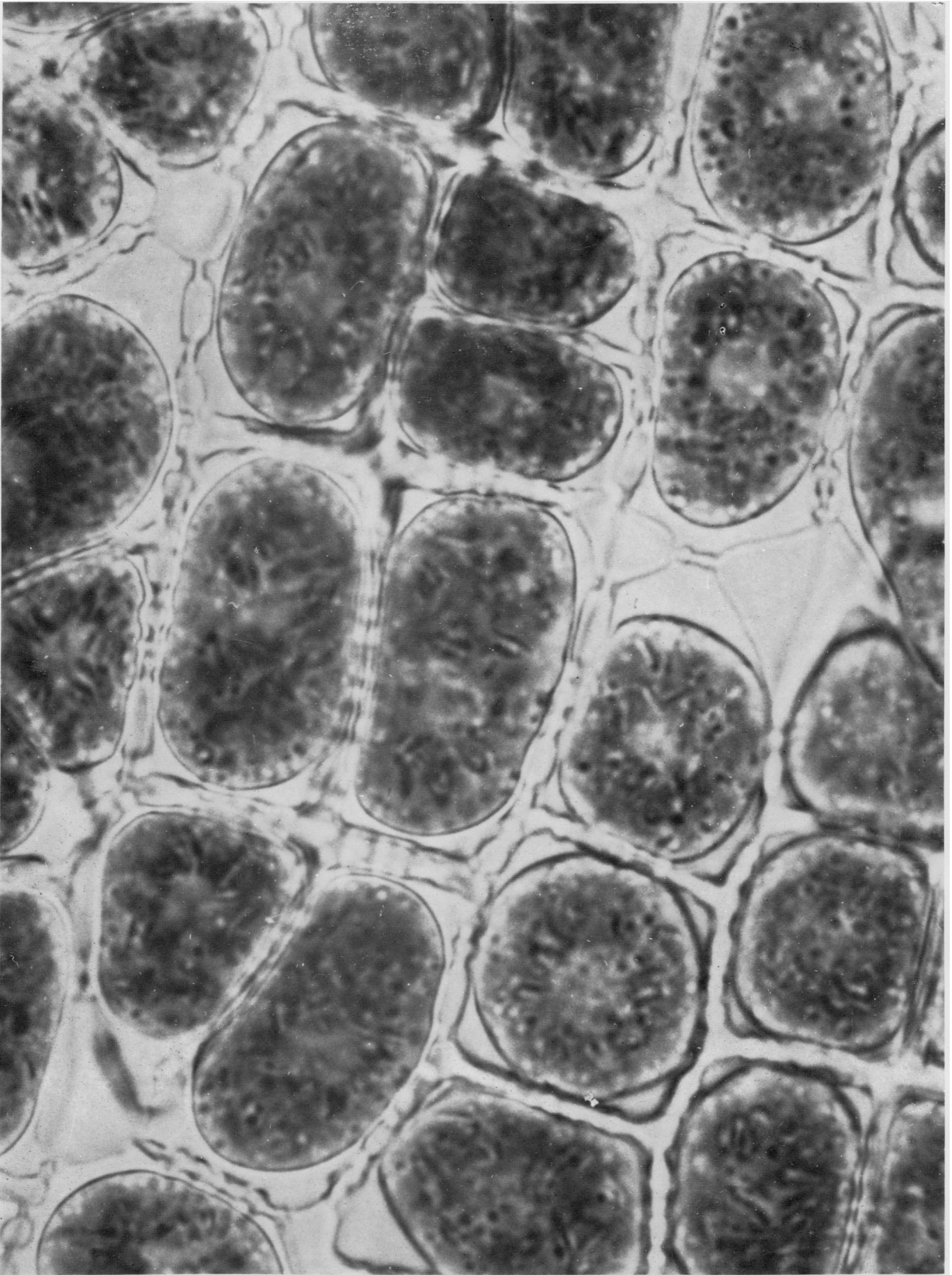


FIG. 1. Cortical cells in a tissue section rapidly immersed into liquid nitrogen and then rewarmed in water at 30°. All of the cells are normally vital stained and plasmolysed. $\times 800$.

Insert

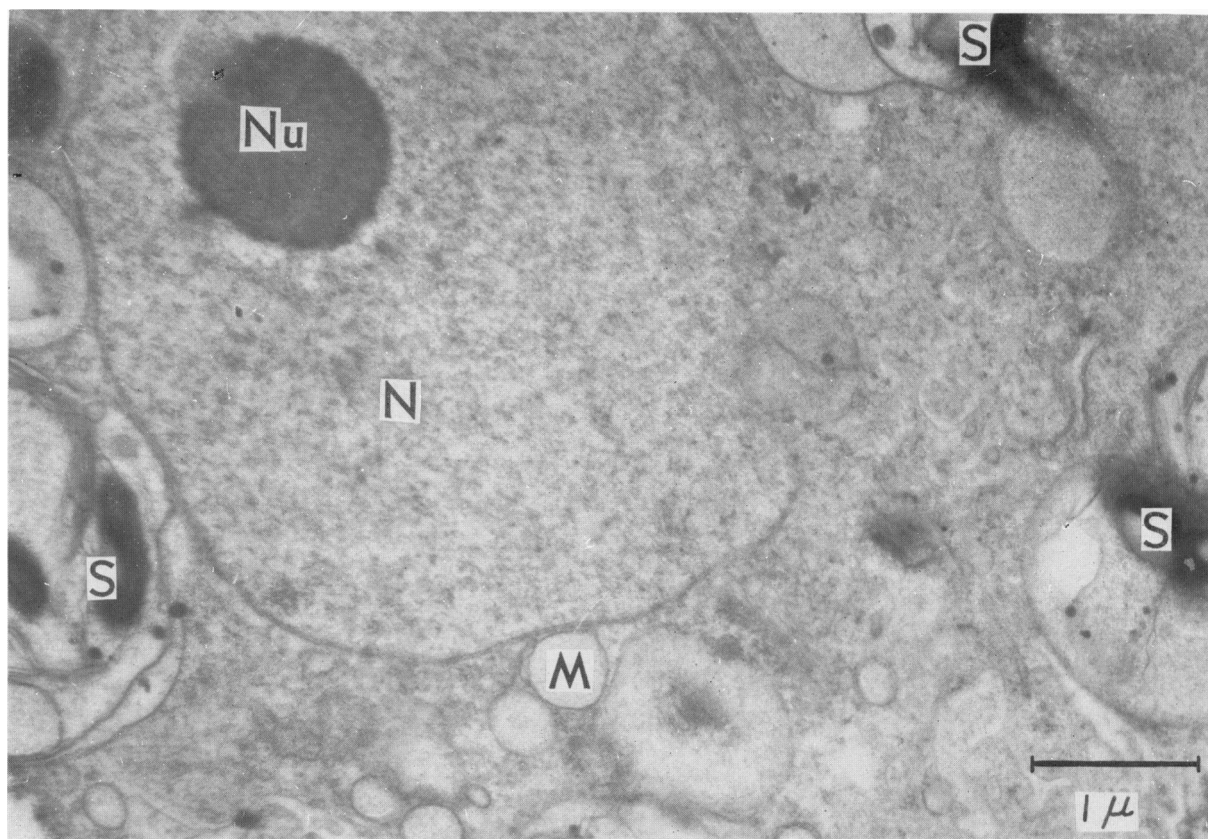


FIG. 2. Electron micrograph of a portion of a nucleus and adjacent cytoplasm of a cortical cell in a controlled, untreated tissue section. Tissue sections were fixed in 2% osmic acid solution at room temperature and then stained with uranyl acetate and lead acetate. N, Nu, M and S represent nucleus, nucleolus, mitochondrion and starch granule respectively. $\times 21,000$.

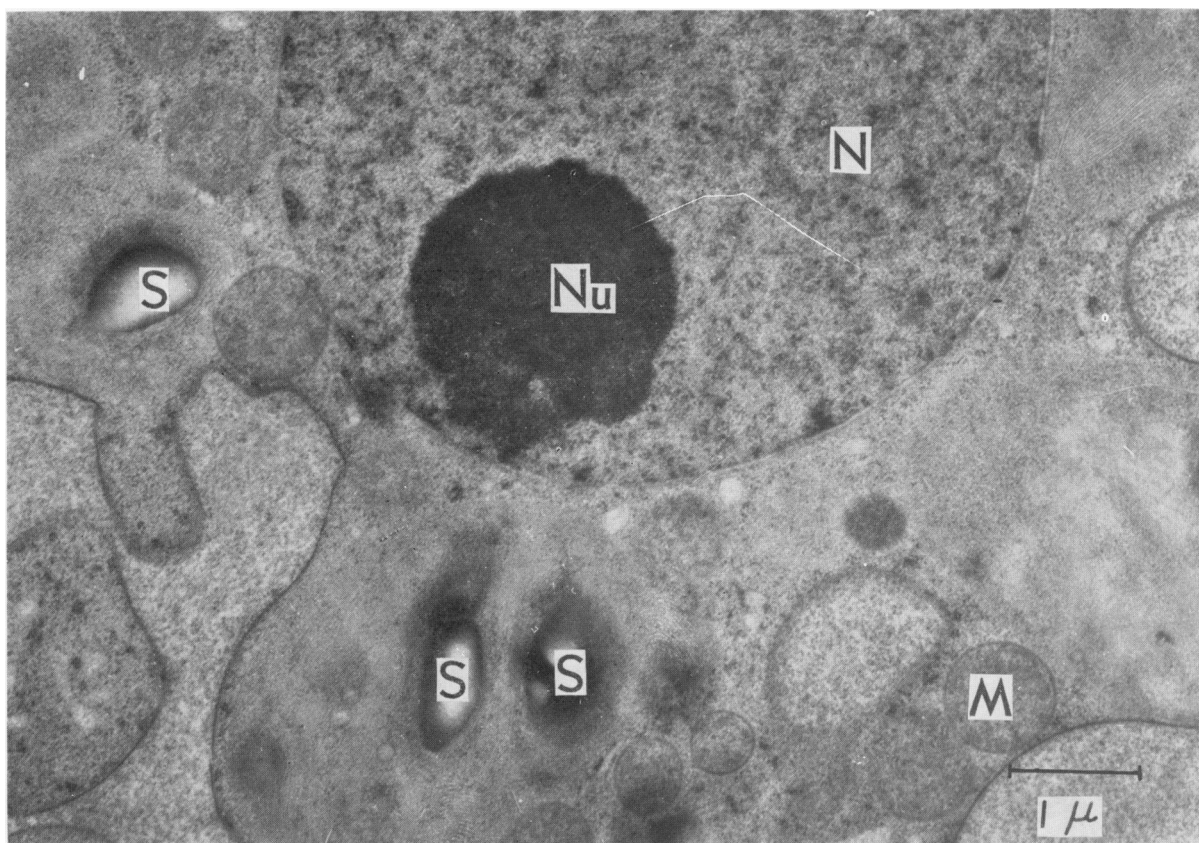


FIG. 3. Electron micrograph of a section of a cortical cell in a tissue section rapidly immersed into liquid nitrogen and freeze-substituted at -78° . No ice cavity and no disturbance by ice formation can be observed in the cells. N, Nu, M and S represent nucleus, nucleolus, mitochondrion and starch granule respectively. $\times 15,000$.

outside the cells. In previous papers (15, 16), however, no direct evidence supporting this hypothetical description was presented. To obtain direct evidence, and especially to clarify the relation between the existence of intracellular ice crystals and cell survival, a further study was made, primarily with the electron microscope.

Materials and Methods

Cortical parenchyma cells from winter twigs of mulberry trees (*Morus bombycis* Koidz.) were used as the experimental materials. Cells from the same twig were used in each series of experiments. This material is very favorable in determining the percentage of survival, because unlike many other trees such as willow, poplar, conifers, etc., when 1 cell layer of cortical tissue is sliced, all of the cells in a tissue section remain alive (fig 1). This tangential tissue sections (20–30 μ thick, 1–2 mm wide, and 2–3 mm long) were sliced from the cortical tissue of a twig using the sharp blade of a straight edge hand razor. These tissue sections were used in each experiment.

To obtain the greatest cooling rate, an unmounted tissue section was held with a forceps and was immersed directly into liquid nitrogen or into an isopentane bath kept at various temperatures. The temperature was determined with 0.1 mm Cu-Co thermocouples and was recorded by an oscilloscope. The cooling rate was usually represented as the time required for the temperature to fall from about -5° to within 5° of the final bath temperature.

The viability of cells was determined by the vital staining test using neutral red and by the plasmolysis test. Plasmolysis and deplasmolysis were repeated twice with a 2-fold isotonic balanced salt solution and water. Normally stained and plasmolysed cells were regarded as normal.

The isopentane bath was cooled at various temperatures ranging from -10 to -110° using a deep-freezer. Small wire net baskets (1.5 cm in diameter, 3 cm in depth) were hung in the baths which were kept at definite temperatures. An unmounted tissue section held with forceps was rapidly immersed into the basket. The basket was then quickly transferred to absolute ethanol at -78° (in a beaker buried in dry-ice-alcohol mixture of -78°). In a -35° cold room, the tissue sections were soon transferred for fixing and freeze-substitution from the ethanol into a solution of 2% osmic acid in absolute ethanol at -78° . The freeze-substitution was continued for 2 or 3 weeks. The materials were then washed twice in absolute ethanol and were embedded in an epoxy resin in the usual way. For great contrast, the materials were stained with uranyl acetate and lead acetate. Normal untreated cortical cells were fixed with an aqueous solution containing 2% osmic acid at room temperature. They were then embedded and stained in the manner described above.

Results

Survival of Tissue Sections Immersed Rapidly in Liquid Nitrogen. To obtain the greatest cooling and rewarming rates, an unmounted tissue section at room temperature was held with a thin forceps and rapidly immersed into liquid nitrogen. The cooling rate was approximately $150,000^\circ$ per minute. The temperature was determined with 0.1 mm Cu-Co thermocouples and was recorded by an oscilloscope. An unmounted tissue section was then rewarmed rapidly in water at 30° or slowly in air at room temperatures. All of the cells survived the rapid rewarming (fig 1), and all were destroyed by the slow rewarming in air. In the unfrozen controls (fig 2) and in the sections which were rapidly immersed into liquid nitrogen from room temperature, no cavities left after ice removal were observed even at a high magnification (fig 3).

Damage Caused During Rewarming Following Removal from Liquid Nitrogen. Damage can occur at any temperature during rewarming following removal from liquid nitrogen. To investigate this problem, tissue sections immersed in liquid nitrogen were rapidly transferred to isopentane baths at temperatures ranging from -10 to -80° at 10° temperature intervals. They were kept there for 10 minutes, 1 hour and 16 hours respectively before rapid rewarming in water at 30° . The results obtained are summarized in figure 4. At the temperatures below -60° , all of the cells remained alive even when kept for 16 hours. Above -50° , survival rates abruptly decreased with the rising temperatures, even in the tissue sections kept for 10 minutes and 1 hour. At -30° , all cells were completely destroyed, even when kept for only 1 minute.

The main characteristic in the survival rates of

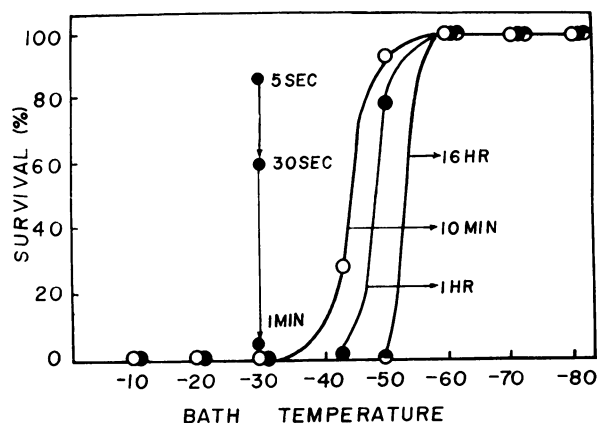


FIG. 4. Survival rates of cells transferred rapidly to isopentane baths kept at various temperatures following removal from liquid nitrogen. Tissue sections were rapidly immersed into liquid nitrogen, and were then immersed into isopentane baths kept at various temperatures for 10 minutes, 1 hour and 16 hours, respectively, following removal from liquid nitrogen, before being rapidly rewarmed in water at 30° .

cells in tissue sections removed from liquid nitrogen and rewarmed in an isopentane bath at -40° is that only the cells at the periphery of a tissue section are destroyed. The cells inside remain alive. This difference is caused by the difference in the rewarming rate between the periphery and the inside.

In the tissue sections kept at -50° or below for 10 minutes and kept at below -60° for 16 hours following their removal from liquid nitrogen, no cavities in the cell sections were observed. In the tissue sections kept at -30° , even when kept only for 10 minutes, many ice cavities were found throughout the cells (fig 5).

Survival Rates of the Cells Immersed Rapidly into Isopentane Baths Kept at Various Temperatures. In view of the above-mentioned results, it may reasonably be considered that if tissue sections can pass rapidly through a temperature range between the freezing point of the tissue and about -60° , the cells suffer no damage. To check obtained evidence on this point, an unmounted tissue section held with a thin forceps was rapidly immersed into isopentane baths cooled at various temperatures. It was kept there for 30 seconds before being rewarmed rapidly in water at 30° . In the temperatures above about -20° , tissue sections were immersed into liquid paraffin to avoid a toxic effect of isopentane. The results obtained are shown in figure 6. The main characteristic of survival is that, in a limited temperature range, survival abruptly decreases or increases from -10° to -30° or from -45° to -60° . In this experiment, an interesting phenomenon in survival of the cells in tissue sections rapidly immersed in isopentane bath at -42° is that only the cells at the periphery of a tissue section remain alive. The inner cells are completely destroyed, because they are cooled at a lower rate than those cells at the periphery.

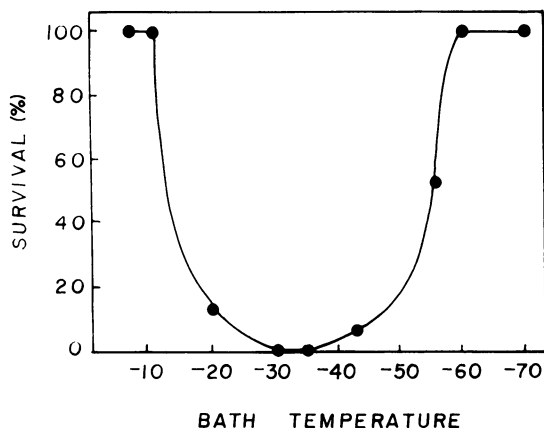


FIG. 6. Survival rates of cortical cells cooled to and rewarmed from various temperatures. A tissue section held with a forceps was rapidly immersed into isopentane or liquid paraffin baths (above -20°) at various temperatures and kept there for 30 seconds before being rewarmed rapidly.

In the tissue sections rapidly immersed in isopentane baths at -10° and at -60° or below for 30 seconds, no ice cavities can be found with the electron microscope in any of the sections; in the sections of the cells rapidly immersed in isopentane bath at -30° , many ice cavities and aborescent structures (the same as those characteristic of ice formations in solutions), can be observed within the cells, even when kept only for 5 seconds (fig 7-1). The ice cavities in the cells kept for 30 seconds at -30° (fig 7-2) are much larger than those kept for 5 seconds at -30° (fig 7-1).

Discussion

One essential point in this study is to ascertain whether or not the cavities seen in the electron micrographs are replicas of the ice particles formed in the initial rapid cooling.

In electron micrographs of unfrozen materials in which, of course, there is no ice, no cavity can be observed. Also, in the outermost parts of the cavities seen in the rapidly cooled cells, no membrane system, characteristic in cellular components can be found, even at a magnification of 30,000. In the tissue sections immersed rapidly in an isopentane bath at -30° for 5 seconds (fig 7-1), a progressive increase is observed in cavity size from the periphery to the inside of a cell. This is apparently because of the difference in the cooling rate. In the periphery, arborescent structures are observed which are the same as cavities characteristic in ice formation in such solutions as gelatin gels (7). From these facts, it may be considered that the cavities in the electron micrographs of the sections of the rapidly cooled cells are the cavities that were left after freeze-substitution of the ice particles formed in the initial rapid cooling. Furthermore, as fixing and freeze-substitution in our experiments are made at -78° , the cavities may be at the sites formerly occupied by the ice, and they may have the general size and orientation of the original ice particle. This result is unlike that of freeze-drying during which the dimensions of the cavities may be increased as a result of post sublimation drying or of recrystallization at the freeze-drying temperature.

In extremely hardy cortical cells of winter twigs of woody plants, it is relatively easy to prevent the cell water from crystallization with rapid cooling and thus to cause them to maintain their viability at super-low temperatures.

The main reasons for the success of the experiment might be attributed to the following characteristics of the cells used: 1) high freezing resistance and, 2) small, thin material, which can be cooled without a suspending medium. The cortical cells in summer twigs of the mulberry tree cannot withstand freezing at -5° and rapid cooling to and rewarming from deep temperatures, even with the same cooling conditions used in winter materials. In the middle of October, the cortical cells of twigs can survive freezing at -5° . The rapid cooling and rewarming

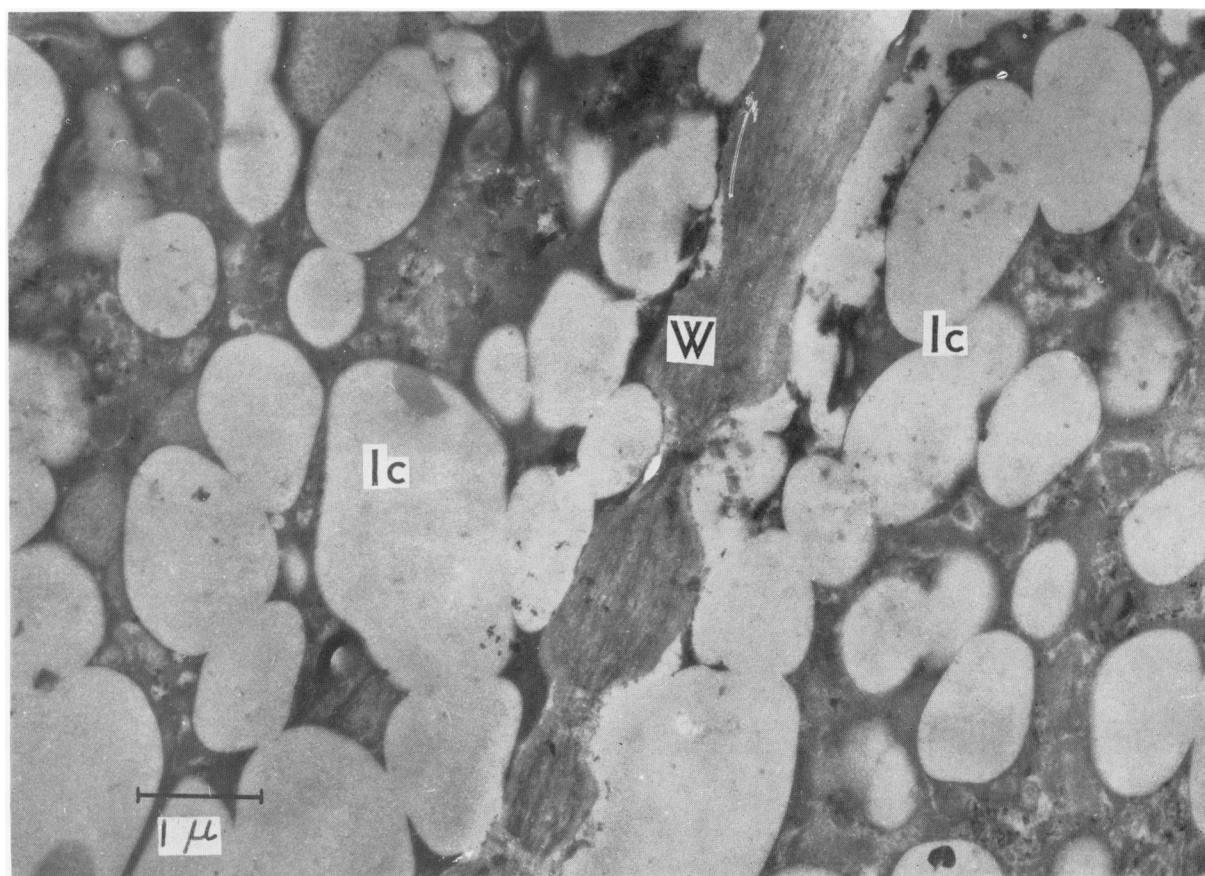


FIG. 5. Electron micrograph of a section of cortical cells kept at -30° for 10 minutes following removal from liquid nitrogen. Many ice cavities (Ic) are observed throughout the cell. Cellular organelle cannot be distinguished. W represents cell wall. $\times 15,000$.

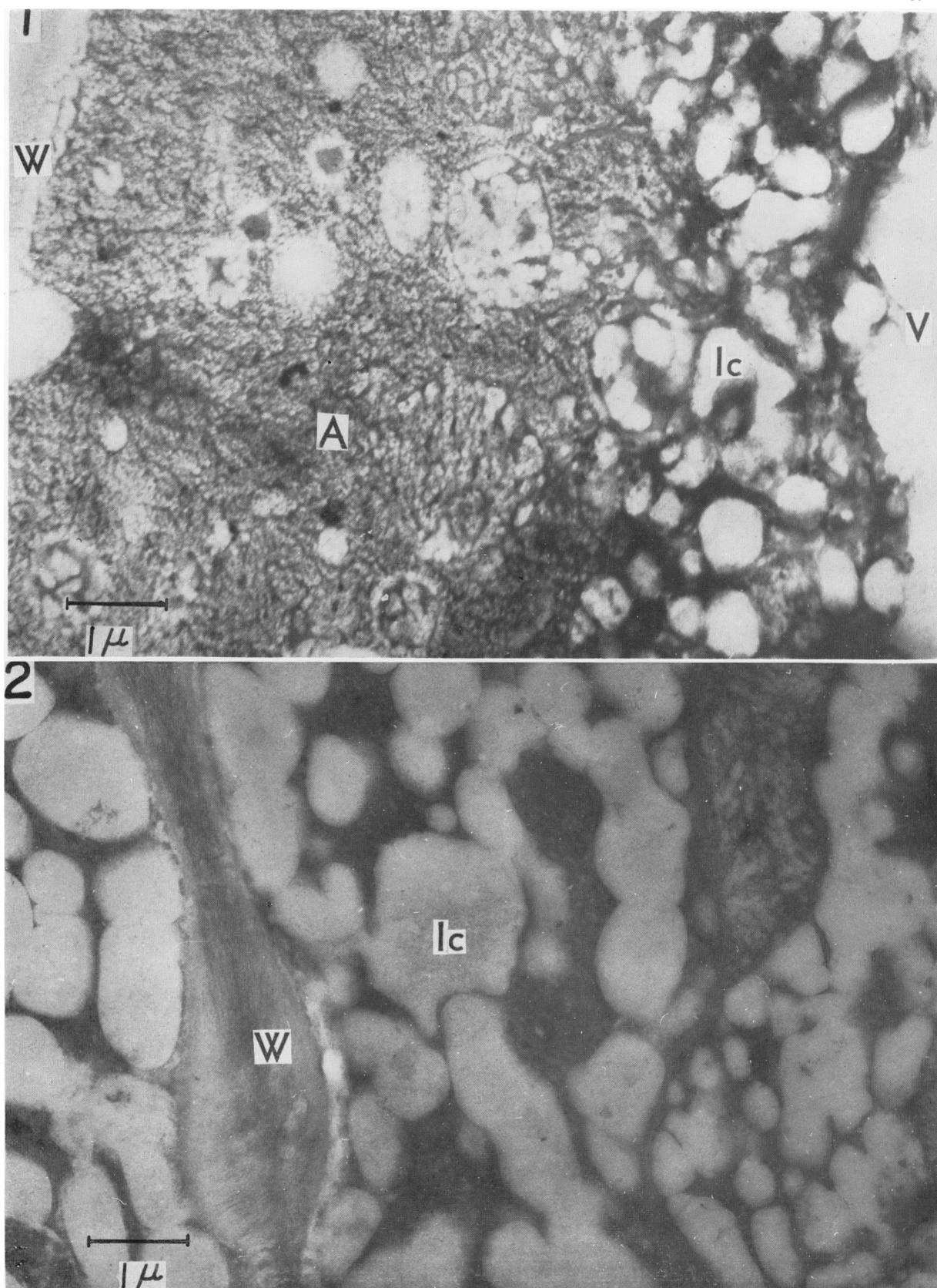


FIG. 7. Electron micrograph of a section of cortical cells rapidly immersed in isopentane at -30° and kept there for 5 seconds (1) or 30 seconds (2) respectively before being freeze-substituted at -78° . Numerous ice cavities (Ic) with different shape and size and aborescent structures (A) are observed within the cell. V and W represent vacuole and cell wall, respectively. $\times 15,000$.

method cannot, however, be successfully applied to this material, unless these cortical cells are previously treated with 2 M solutions of dimethyl sulfoxide, ethylene glycol and sugars. In this reason, after hardening at 0° for 10 days, the cortical cells became resistant to freezing at -20°. They could survive rapid immersion into liquid nitrogen and subsequent rapid rewarming in water at 30°.

From these facts, it is apparent that the application of the rapid cooling and rewarming method for maintaining the viability of cells at super-low temperatures seems to be limited to the materials with high freezing resistance. For this reason, there is limited application of the method for the less hardy materials used chiefly by many cryobiologists, even if the materials could be ultra rapidly cooled and rewarmed (1, 10).

The hardy cortical cells in the twigs of mulberry tree have a high content of sugar, which contributes to the prevention or retardation of injurious intracellular crystallization that occurs during rapid cooling to super-low temperatures and that results in lessening the damage to the cells. It may be considered that another mechanism, pointed out by Tumanov and Krasavtsev (17, 18), may be operative which can protect water in cells from crystallization during rapid cooling. This may be a structural change in the protoplast produced during hardening. Further studies are required to clarify this mechanism.

In the tissue sections that were immersed into isopentane at -30°, all of the cells were completely destroyed, even after a subsequent rapid rewarming. In the tissue sections that were immersed in isopentane at about -40°, only the cells at the periphery of a tissue section retained their viability. The inner cells were completely destroyed. From this evidence it can be said that the cooling rate at the periphery may be greater than that on the inside of the same tissue section. Further, the fact that all of the cells are alive in the tissue sections immersed in isopentane at temperatures below -60°, indicates that the increase in the cooling rate results in a progressive increase in survival.

In the process of rewarming following removal from liquid nitrogen, the opposite phenomena were observed. In tissue sections transferred from liquid nitrogen to -40° for 10 minutes, only the cells at the periphery of a tissue section were selectively destroyed, while the cells on the inside remained alive. In the tissue sections transferred to -50° following removal from liquid nitrogen, almost all of the cells were alive, however, when they were subsequently transferred to -30° for 1 minute, all were destroyed. These facts and the results obtained with the electron microscope support the idea that the growth of recrystallization nuclei takes place in the cells during the process of a slow rewarming following a rapid cooling to deep temperatures.

The survival curves presented in this paper may be explained in terms of the growth rate of intracellular ice crystals. At low temperatures below

-60°, the growth rate of ice crystals is so small that the ice formed in the cells during rapid cooling may be small enough to be innocuous, even when exposed for 16 hours. When rewarming is carried out rapidly, they melt before they can grow, and all cells remain viable. At the temperatures above -40°, the growth rate may be great, because the ice formed is large enough to be immediately damaging. We can conclude that in the tissue sections immersed rapidly in the isopentane bath at temperatures from -20° to -40° or in those rewarmed at the same temperatures following removal from liquid nitrogen, survival remains very low, even with subsequent rapid rewarming.

For these reasons, the basic factor in maintaining viability with rapid cooling and rewarming is not whether ice is formed within the cells, but rather the size of the ice crystals. To determine the damaging size of intracellular ice crystals, further studies are being made.

Literature Cited

1. KOJIMA, Y. 1965. Freezing injury in rabbit erythrocytes. Morphology. *Low Temp. Sci. Ser. B* 23: 129-48.
2. LUYET, B. J. 1937. The vitrification of organic colloids and of protoplasts. *Biodynamica* 1: 1-14.
3. LUYET, B. J. AND P. M. GEHENIO. 1938. The survival of moss vitrified in liquid air and its relation to water content. *Biodynamica* 2: 1-7.
4. LUYET, B. J. AND P. M. GEHENIO. 1954. Effect of the rewarming velocity on the survival of embryonic tissues frozen after treatment in ethylene glycol. *Biodynamica* 7: 213-23.
5. LUYET, B. J. AND G. RAPATZ. 1958. Patterns of ice formation in some aqueous solutions. *Biodynamica* 8: 1-68.
6. LUYET, B. J. 1960. On various phase transitions occurring in aqueous solutions at low temperatures. *Ann. N. Y. Acad. Sci.* 85: 549-69.
7. MACKENZIE, A. P. AND B. J. LUYET. 1962. Electron microscope study of the structure of very rapidly frozen gelatine solutions. *Biodynamica* 9: 47-69.
8. MENS, L. J. AND B. J. LUYET. 1961. An electron microscope study of the distribution of ice in single muscle fibers frozen rapidly. *Biodynamica* 8: 261-94.
9. RAPATZ, G., J. NATH, AND B. J. LUYET. 1963. Electron microscope study of erythrocytes in rapidly frozen mammalian blood. *Biodynamica* 9: 83-94.
10. RAPATZ, G. AND B. J. LUYET. 1965. Effect of cooling rate on the preservation of erythrocytes in frozen blood containing various protective agents. *Biodynamica* 9: 333-50.
11. SAKAI, A. 1956. Survival of plant tissue at super-low temperatures. *Low Temp. Sci. Ser. B* 14: 17-23.
12. SAKAI, A. 1958. Survival of plant tissue at super-low temperatures II. *Low Temp. Sci. Ser. B* 16: 41-53.

13. SAKAI, A. 1960. Survival of the twig of woody plants at -196° . *Nature* 85: 392-94.
14. SAKAI, A. 1965. Survival of plant tissue at super-low temperatures III. Relation between effective prefreezing temperatures and degree of frost hardness. *Plant Physiol.* 40: 882-87.
15. SAKAI, A. 1966. Survival of plant tissue at super-low temperatures IV. Cell survival with rapid cooling and rewarming. *Plant Physiol.* 41: 1050-54.
16. SAKAI, A. 1967. Survival of plant tissue at super-low temperatures by rapid cooling and rewarming. *Cellular Injury and Resistance in Freezing Organisms* (E. Asahina, ed.) *Low Temp. Sci. Ser. A Sapporo*: 119-30.
17. TUMANOV, I. I. AND O. A. KRASAVTSEV. 1966. Elucidation of the conditions required for vitrification of plant cells. *Plant Physiol. USSR.* 13: 144-54.
18. TUMANOV, I. I. AND O. A. KRASAVTSEV. 1966. A study of the mechanism of vitrification of water in plant cells. *Plant Physiol. USSR.* 13: 487-93.